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IL-2, -7, and -15, but Not Thymic Stromal Lymphopoeitin, Redundantly Govern CD4$^+$Foxp3$^+$ Regulatory T Cell Development[1]

Kieng B. Vang, Jianying Yang, Shawn A. Mahmud, Matthew A. Burchill,[2] Amanda L. Vegoe, and Michael A. Farrar[3]

Common γ chain (γc)-receptor dependent cytokines are required for regulatory T cell (Treg) development as $\gamma c^{-/-}$ mice lack Tregs. However, it is unclear which γc-dependent cytokines are involved in this process. Furthermore, thymic stromal lymphopoietin (TSLP) has also been suggested to play a role in Treg development. In this study, we demonstrate that developing CD4$^+$Foxp3$^+$ Tregs in the thymus express the IL-2Rβ, IL-4Rα, IL-7Rα, IL-15Rα, and IL-21Rα chains, but not the IL9Rα or TSLPRα chains. Moreover, only IL-2, and to a much lesser degree IL-7 and IL-15, were capable of transducing signals in CD4$^+$Foxp3$^+$ Tregs as determined by monitoring STAT5 phosphorylation. Likewise, IL-2, IL-7, and IL-15, but not TSLP, were capable of inducing the conversion of CD4$^+$CD25$^+$Foxp3$^-$ thymic Treg progenitors into CD4$^+$Foxp3$^+$ mature Tregs in vitro. To examine this issue in more detail, we generated *IL-2R$\beta^{-/-}$* × *IL-7R$\alpha^{-/-}$* and *IL-2R$\beta^{-/-}$* × *IL-4R$\alpha^{-/-}$* mice. We found that *IL-2R$\beta^{-/-}$* × *IL-7R$\alpha^{-/-}$* mice were devoid of Tregs thereby recapitulating the phenotype observed in $\gamma c^{-/-}$ mice; in contrast, the phenotype observed in *IL-2R$\beta^{-/-}$* × *IL-4R$\alpha^{-/-}$* mice was comparable to that seen in *IL-2R$\beta^{-/-}$* mice. Finally, we observed that Tregs from both *IL-2$^{-/-}$* and *IL-2R$\beta^{-/-}$* mice show elevated expression of IL-7Rα and IL-15Rα chains. Addition of IL-2 to Tregs from *IL-2$^{-/-}$* mice led to rapid down-regulation of these receptors. Taken together, our results demonstrate that IL-2 plays the predominant role in Treg development, but that in its absence the IL-7Rα and IL-15Rα chains are up-regulated and allow for IL-7 and IL-15 to partially compensate for loss of IL-2. ***The Journal of Immunology,*** **2008, 181: 3285–3290.**

CD4$^+$Foxp3$^+$ regulatory T cells (Tregs)[4] that develop in the thymus play a key role in preventing autoimmune disease (1). Multiple signals are required to induce the development of Tregs. These include signals emanating from the TCR and the costimulatory molecule CD28 (2–5). In addition, several studies have suggested that cytokines play a key role in this process (6–11). However, which cytokines are involved has remained controversial. For example, it has been known for many years that mice lacking IL-2, or the IL-2Rα or IL-2Rβ chains, develop lethal autoimmune disease (12–14). This was initially attributed to defective Treg development as these mice lacked CD4$^+$CD25$^+$ T cells. More recent studies using the transcription factor Foxp3 as an identifier of Tregs found that young *IL-2$^{-/-}$* and *IL-2R$\alpha^{-/-}$* mice have relatively normal numbers of CD4$^+$Foxp3$^+$ Tregs (8, 15, 16). In contrast, both *IL-2R$\beta^{-/-}$* and *IL-2$^{-/-}$* × *IL-15$^{-/-}$* mice exhibited significant decreases in Treg numbers, suggesting that IL-2 and IL-15 play a redundant role in Treg development (8, 9).

Importantly, although Treg differentiation is inhibited in *IL-2R$\beta^{-/-}$* mice, Tregs are not completely absent (8, 9). This raises the possibility that other cytokines can also drive Treg differentiation. Along these lines, Watanabe et al. (17) have suggested a role for the cytokine thymic stromal lymphopoietin (TSLP). Specifically, they suggested that TSLP production by Hassall's corpuscles plays an important role in human Treg development (17). Likewise, the common γ-chain (γc), which is closely related to the TSLPR, has also been shown to be involved in Treg development. For example, we and others have demonstrated that mice lacking γc are devoid of Tregs (8, 15). The γc forms a component of multiple cytokine receptors including those for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (18). Thus, two key questions in Treg development are 1) which γc-dependent cytokines can induce Treg development and 2) whether TSLP signals are also involved in this process. In this study, we demonstrate that developing Tregs express IL-2Rβ, IL-7Rα and IL-15Rα and respond to IL-2, IL-7, and to a much lesser degree IL-15, by inducing STAT5 activation in CD4$^+$Foxp3$^+$ thymocytes. Similarly, IL-2-induced conversion of CD4$^+$CD25$^+$Foxp3$^-$ thymic Treg progenitors into CD4$^+$Foxp3$^+$ mature Tregs. IL-7 and IL-15 also induced conversion of thymic Treg progenitors into mature CD4$^+$Foxp3$^+$ Tregs, albeit much less effectively; in contrast, TSLP showed no activity in this conversion assay. IL-4 signaling also does not appear to play a role in Treg development as *IL-4R$\alpha^{-/-}$* × *IL-2R$\beta^{-/-}$* mice show comparable numbers of Tregs as that seen in *IL-2R$\beta^{-/-}$* mice. In contrast, *IL-2R$\beta^{-/-}$* × *IL-7R$\alpha^{-/-}$* mice exhibit a developmental block which mimics that seen in $\gamma c^{-/-}$ mice. Finally, the

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[4] Abbreviations used in this paper: Treg, regulatory T cell; γc, common gamma chain; p-STAT5, phospho-STAT5; LMC, littermate control; TSLP, thymic stromal lymphopoietin.

expression of IL-7Rα and IL-15Rα chains is suppressed in mature Tregs; this suppression does not occur in *IL-2*$^{-/-}$ or *IL-2R*$\beta^{-/-}$ mice, demonstrating that IL-7Rα and IL-15Rα down-regulation occurs via an IL-2/IL-2Rβ-dependent signaling pathway. Our findings demonstrate that IL-2, IL-7, and IL-15, are the critical γc-dependent cytokines that are responsible for promoting Treg development. In contrast, developing Tregs do not express the TSLPRα-chain, nor respond to TSLP, by inducing phospho-STAT5 (p-STAT5). Thus, at least in the mouse, TSLP does not appear to play a direct role in Treg development.

Materials and Methods

Mice

IL-2$^{-/-}$, *IL-2R*$\beta^{-/-}$, *IL-7R*$\alpha^{-/-}$, and *IL-4R*$\alpha^{-/-}$ mice were obtained from The Jackson Laboratories. *IL-2R*$\beta^{-/-}$, *IL-7R*$\alpha^{-/-}$, and *IL-4R*$\alpha^{-/-}$ mice were crossed in our laboratory to obtain *IL-2R*$\beta^{-/-}$ $\times$ *IL-7R*$\alpha^{-/-}$ and *IL-4R*$\alpha^{-/-}$ $\times$ *IL-2R*$\beta^{-/-}$ mice. Mice used were on a C57BL/6 background with the exception of the *IL-4R*$\alpha^{-/-}$ and *IL-4R*$\alpha^{-/-}$ $\times$ *IL-2R*$\beta^{-/-}$ mice, which were on a mixed BALB/c $\times$ C57BL/6 background, and Foxp3-GFP reporter mice, which were on a mixed C57BL/6 $\times$ 129 background. Foxp3-GFP reporter mice were provided by Dr. Sasha Rudensky (University of Washington School of Medicine, Department of Immunology, Seattle, WA).

Flow cytometry and FACS analysis

Mice were sacrificed and lymph node, spleen, and thymus were isolated. Five million cells were used per staining condition. Cells were first pretreated with an Ab that blocks Fc receptor binding (Clone 24G2). Cells were subsequently stained with the following Abs from eBioscience: CD4-Alexa 700, CD8-allophycocyanin-Alexa Fluor 750 or CD8-FITC, CD3ε-FITC, CD25-PE-Cy7 (PE-Cy 7), or CD25-allophycocyanin. In addition, biotinylated Abs for CD122 (IL2Rβ), CD124 (IL4Rα), CD127 (IL7Rα), and IL21Rα, were obtained from eBioscience. Abs for IL-9Rα, TSLPRα, and IL-15Rα were obtained from R&D Systems. Isotype control Abs for TSLPRα and IL-15Rα were obtained from R&D Systems, while isotype controls for IL-9Rα were obtained from eBioscience. Both the IL-9Rα and TSLPRα were biotinylated according to the manufacturer's instructions (Sigma-Aldrich, Cat. no. BTAG-1KT), while the IL-15Rα Ab was purchased in a biotinylated form. Streptavidin allophycocyanin from eBioscience was used as a secondary reagent to reveal staining with biotinylated Abs. Intracellular Foxp3 staining was done after fixation, permeabilization, and overnight incubation at 4°C as described previously (8).

To examine whether IL-2 alters IL-7Rα and IL-15Rα expression on Tregs in *IL-2*$^{-/-}$ mice, we purified CD4$^+$ splenocytes from *IL-2*$^{-/-}$ mice by MACS beads enrichment (Miltenyi Biotec). Purified cells were then stimulated with IL-2 (100 U/ml) for 4, 8, 12, and 24 h. Cells were then harvested and stained for IL-7Rα, IL-15Rα, and Foxp3 expression and analyzed on an LSR II flow cytometer (BD Biosciences).

Flow cytometry for p-STAT5

Single-cell suspensions were generated from isolated spleens and thymii from Foxp3-GFP reporter mice. These cell suspensions were pretreated with an Ab that blocks Fc receptor binding. Cells were then stained for the surface markers CD4, CD8, and CD25. Five million cells were then serum starved in 500 μl of 1$\times$ DMEM for 30 min at 37°C, then stimulated with either 100 U/ml of IL-2 (PeproTech), or 50 ng/ml IL-4 (PeproTech), IL-7 (R&D Systems), IL-9 (R&D Systems), TSLP (R&D Systems), IL-15 (R&D Systems), or IL-21 (PeproTech) for 20 min. After stimulation, the cells were washed with 1$\times$ DMEM to remove all traces of the supernatant; they were then resuspended in 100 μl of fixation medium from the Caltag Fix and Perm kit and incubated at 37°C for 15 min. Afterward, 1 ml of 4°C 100% methanol was added and the cells were incubated overnight at 4°C in the dark. Intracellular p-STAT5 staining was done using the Caltag Fix and Perm kit and PE-conjugated anti-p-STAT5 (BD Biosciences). Non-stimulated cells were used as negative controls.

Treg conversion assay

The conversion assay of Treg progenitors into CD4$^+$Foxp3$^+$ Tregs was conducted as previously described (10). In brief, CD4$^+$CD25$^+$Foxp3$^-$ Treg progenitors were sorted from Foxp3-GFP reporter mice (19) and placed in culture in the presence of the indicated amounts of cytokine. Twenty-four hours later cells were stained for CD4 and CD25 and analyzed for expression of these markers plus Foxp3-GFP using an LSR II flow cytometer.

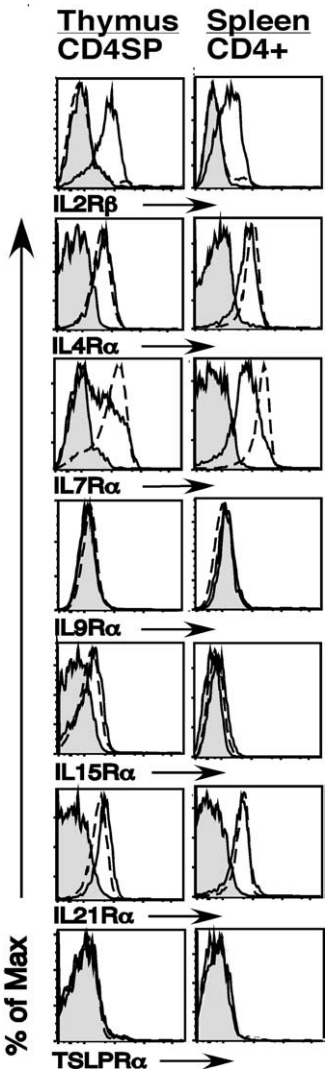

FIGURE 1. γc cytokine-family receptor expression on CD4$^+$Foxp3$^+$ single positive thymocytes and CD4$^+$Foxp3$^+$ splenocytes. Thymus and spleen cells from 5- to 9-wk-old C57BL/6 mice were harvested and stained with Abs to CD4, CD8, and Foxp3, to identify distinct thymic and splenic T cell subsets, as well as with Abs to IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα, IL-21Rα, and TSLPRα. Shown are flow cytometry histograms of CD4 single positive (SP) thymocytes (*left column*) and CD4$^+$ splenocytes (*right column*). Gray filled in histograms represent staining of the corresponding CD4$^+$Foxp3$^+$ population with isotype control Ab. Solid lines and broken lines represent staining of CD4$^+$Foxp3$^+$ and CD4$^+$Foxp3$^-$ cells, respectively. A representative example of four independent experiments is depicted (n = 17).

Results

To examine the role that different cytokines play in Treg development in the thymus, we first analyzed the expression of IL-2Rβ, IL-4Rα, IL-7Rα, IL-9Rα, IL-15Rα, IL-21Rα, and TSLPRα on CD4$^+$Foxp3$^+$ thymocytes and CD4$^+$Foxp3$^+$ splenocytes. We found four basic patterns of cytokine receptor expression. First, the IL-2Rβ-chain was selectively expressed on CD4$^+$Foxp3$^+$ thymocytes (Fig. 1). This pattern of expression was maintained in splenic Foxp3$^+$ vs Foxp3$^-$ T cells. Second, IL-9Rα and TSLPRα were not observed on either Foxp3$^+$ or Foxp3$^-$ thymocytes. To confirm that our staining for these receptors was working, we also stained peritoneal B1 B cells and CD19$^+$ pre-B cells, which have previously been reported to express the IL-9Rα and TSLPRα chains,

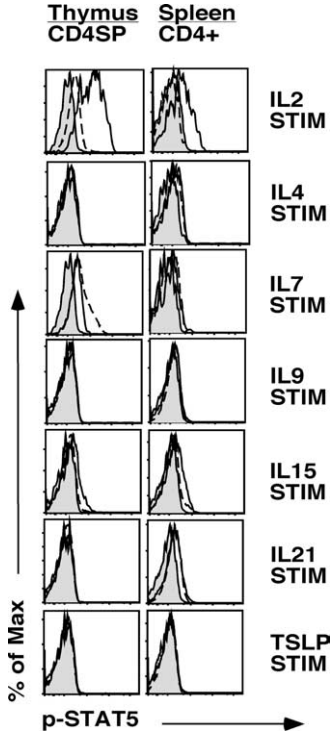

FIGURE 2. Cytokine stimulation and phospho-STAT5 expression on $CD4^+Foxp3^+$ thymocytes and splenocytes. Single-cell suspensions of thymocytes or splenocytes from Foxp3-GFP mice were serum starved for 30 min and then stimulated with IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, or TSLP for 20 min. Cells were then stained with Abs to CD4, CD8, CD25, and phospho-STAT5 as described in the methods section. Shown are histograms of phospho-STAT5 expression in CD4SP thymocytes (*left column*) and $CD4^+$ splenocytes (*right column*). Gray filled in histograms represent staining of unstimulated Foxp3-GFP^+ cells. Solid lines and broken lines represent staining of stimulated Foxp3-GFP^+ and Foxp3-GFP^- cells, respectively. A representative example of two independent experiments is depicted ($n = 3$ mice). Similar results were obtained when using CD25 to identify Tregs in C57BL/6 mice ($n = 13$ mice, data not shown).

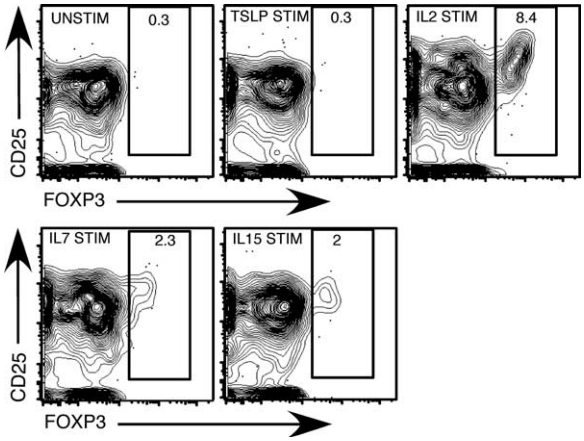

FIGURE 3. IL-2, IL-7, and IL-15, but not TSLP, induce the conversion of $CD4^+CD25^+Foxp3^-$ thymic Treg progenitors into $CD4^+Foxp3^+$ Tregs. Sorted $CD4^+CD25^+$ $Foxp3^-$ thymic Treg progenitors from Foxp3-GFP reporter mice were stimulated with 10 U/ml IL-2, 5 ng/ml IL-7, 100 ng/ml IL-15, and 50 ng/ml TSLP in culture overnight. After 24 h of stimulation, cells were stained with Abs to CD4 and CD25. Shown are contour plots of Foxp3 vs CD25 for CD4-gated cells (95% of cells were $CD4^+$) after stimulation with medium alone (*top left*), TSLP (*top middle*), IL-2 (*top right*), IL-7 (*bottom left*), or IL-15 (*bottom right*). A representative example of three independent experiments is depicted.

respectively (17, 18). We detected IL-9Rα expression on peritoneal B1 B cells; as expected, we also observed TSLPRα expression on pre-B cells in the bone marrow, thereby indicating that our Abs to IL9Rα and TSLPRα are capable of detecting expression of these receptors (data not shown). Third, IL-4Rα and IL-21Rα were expressed equally on $Foxp3^+$ vs $Foxp3^-$ thymocytes and splenic T cells (Fig. 1). Last, we observed a dynamic expression pattern for the IL-7Rα and IL-15Rα chains. Expression of both of these receptors was observed in $CD4^+Foxp3^+$ thymocytes, but was significantly reduced in splenic Tregs (Fig. 1). Thus multiple γc-dependent cytokine receptors, but not the TSLPRα-chain, are expressed on developing Tregs in the thymus.

To assess whether receptor expression on Tregs correlated with function, we stimulated cells with IL-2, IL-4, IL-7, IL-9, IL-15, IL-21, and TSLP and examined STAT5 activation by intracellular staining for p-STAT5. We focused on STAT5 as previous reports have indicated that STAT5 plays a critical role in Treg development (8, 10, 20). For these studies, we identified Tregs using Foxp3-GFP reporter mice; similar studies were also conducted using CD25 as a marker of Tregs. We identified three distinct response patterns. First, IL-2 induced robust STAT5 activation in $CD4^+Foxp3^+$ thymocytes; splenic Tregs remained highly responsive to IL2 stimulation (Fig. 2). Second, IL-7 and IL-15 induced modest STAT5 activation in $CD4^+Foxp3^+$ thymocytes. However, these responses were almost completely eliminated in $CD4^+$ $Foxp3^+$ splenocytes (Fig. 2). Third, IL-4, IL-9, IL-21, and TSLP did not induce detectable STAT5 activation on $CD4^+Foxp3^+$ thymocytes (Fig. 2). Similar results were obtained when gating on $CD4^+CD25^+$ T cells, with the exception that under those staining conditions IL-4 led to very weak STAT5 phosphorylation in $CD4^+Foxp3^+$ thymocytes and splenocytes (data not shown). A potential caveat with the IL-15 studies is that IL-15 could be presented by the IL-15Rα-chain via trans presentation in vivo (21). It is possible, therefore, that our ex vivo stimulation studies may not have allowed for optimal transpresentation of IL-15 to $CD4^+Foxp3^+$ Tregs. Thus, developing thymic Tregs respond to IL-2 and IL-7, and to a lesser degree IL-15.

Previous reports have suggested that TSLP plays a role in Treg development (22). However, *TSLPR*$^{-/-}$ mice have no reported defects in Treg develoment or function (23). This latter observation is consistent with our failure to observe TSLPR expression on developing Tregs. A potential caveat with our studies is that TSLPR expression levels may be below the limits of detection by flow cytometery. Likewise, the amount of STAT5 phosphorylation induced by TSLP might be below the level that we can detect by flow cytometry. To explore this in more detail, we examined whether TSLPR mRNA was detectable by RT-PCR in $CD4^+$Foxp3-GFP^+ thymcoytes. These studies indicated that TSLPR mRNA could be detected at some level in developing Tregs (data not shown). A key question then is whether this results in expression of a receptor capable of inducing biological responses. To address this question, we made use of the recent identification of $CD4^+CD25^+Foxp3^-$ thymocytes as penultimate Treg progenitors that can be converted into $CD4^+Foxp3^+$ mature Tregs following stimulation with IL-2 (10, 11). For these studies, we isolated $CD4^+CD25^+$Foxp3-GFP^- Treg progenitors and stimulated them overnight with either IL-2, IL-7, IL-15, or TSLP. The cultured cells were then examined for Foxp3-GFP expression. As shown in Fig. 3, IL2 induced clear conversion of Treg progenitors into $CD4^+$Foxp3-GFP^+ Tregs. IL-7 and IL-15 were also capable of inducing the conversion of Treg progenitors into $Foxp3^+$ Tregs, although they were much less effective than IL-2. In contrast,

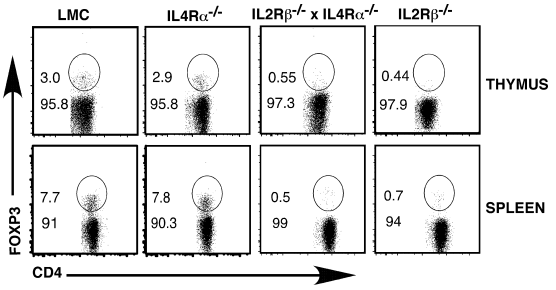

FIGURE 4. Treg development in *IL-4Rα*$^{-/-}$ and *IL-2Rβ*$^{-/-}$ × *IL-4Rα*$^{-/-}$ mice. Thymus and spleen were harvested from 4- to 5-wk-old LMC, *IL-4Rα*$^{-/-}$, *IL-2Rβ*$^{-/-}$, and *IL-2Rβ*$^{-/-}$ × *IL-4Rα*$^{-/-}$ mice. Cells were stained with Abs to CD4, CD8, and Foxp3 to identify Tregs. Shown are flow cytometry plots of thymus (*top panel*) or spleen cells (*bottom panel*) gated on CD4$^+$ T cells. A representative example of six independent experiments is depicted.

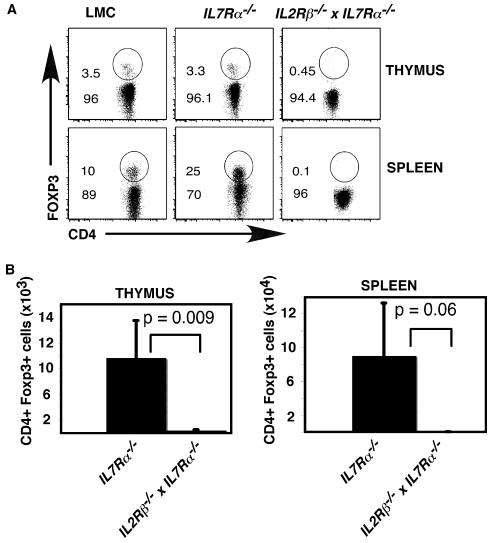

FIGURE 5. Treg development in *IL-7Rα*$^{-/-}$ and *IL-2Rβ*$^{-/-}$ × *IL-7Rα*$^{-/-}$ mice. *A*, Thymus and spleen were harvested from 4- to 5-wk-old LMC, *IL-7Rα*$^{-/-}$, and *IL-2Rβ*$^{-/-}$ × *IL-7Rα*$^{-/-}$ mice. Cells were stained with Abs to CD4, CD8, and Foxp3 to identify Tregs. Shown are flow cytometry plots of thymus (top panel) or spleen cells (*bottom panel*) gated on CD4$^+$ T cells. A representative example of six independent experiments is depicted. *B*, Shown are bar graphs representing total numbers of CD4$^+$Foxp3$^+$ Tregs in the thymus (*left panel*) and spleen (*right panel*). Error bars represent SEM; n = 6 *IL-7Rα*$^{-/-}$ and 6 *IL-2Rβ*$^{-/-}$ × *IL-7Rα*$^{-/-}$ mice; p values were calculated using two-tailed Student's t test.

TSLP stimulation failed to induce conversion of any Treg progenitors into Foxp3$^+$ Tregs. Thus, even if the TSLPR is expressed at very low levels on developing Tregs, it is incapable of inducing Treg differentiation following stimulation with TSLP.

Our observation that IL-2Rβ and IL-7Rα were the predominant receptors expressed on developing thymocytes suggested that IL-2, IL-7, and IL-15 were most likely the key γc-dependent cytokines that drive Treg development. However, given the expression of the IL-4Rα-chain on developing Tregs, we examined whether IL4-dependent signals also played a role in this process. *IL-4Rα*$^{-/-}$ mice show no decrease in the percentage of Tregs in the thymus relative to littermate control (LMC) mice (Fig. 4). Furthermore, splenic Tregs were also not reduced in *IL-4Rα*$^{-/-}$ mice (Fig. 4). To examine whether IL-2Rβ and IL-4Rα-dependent signals played a redundant role in Treg development, we generated *IL-2Rβ*$^{-/-}$ × *IL-4Rα*$^{-/-}$ mice. As previously reported, *IL-2Rβ*$^{-/-}$ mice exhibited reduced numbers of Tregs in both the thymus and spleen; a further reduction was not observed in *IL-2Rβ*$^{-/-}$ × *IL-4Rα*$^{-/-}$ mice (Fig. 4). These findings strongly suggest that IL-4Rα-dependent signals are not required for Treg development. It is important to note here that unlike our previous studies, which used *IL-2Rβ*$^{-/-}$ mice on the C57BL/6 background, the *IL-4Rα*$^{-/-}$ and *IL-2Rβ*$^{-/-}$ × *IL-4Rα*$^{-/-}$ mice in these experiments are on a mixed C57BL/6 × BALB/c background. We have consistently noticed that the *IL-2Rβ*$^{-/-}$ mice on the C57BL/6 × BALB/c background mice have a more severe phenotype (i.e., fewer Tregs at an earlier age) than *IL-2Rβ*$^{-/-}$ on the C57BL/6 background. This results in *IL-2Rβ*$^{-/-}$ mice on the mixed background having a reduced percentage of Tregs relative to that seen in *IL-2Rβ*$^{-/-}$ mice on the C57BL/6 background.

Given the expression of both functional IL-7Rα and IL-2Rβ on developing Tregs, we predicted that these two cytokine receptors might both be capable of driving Treg development. Consistent with our previous report, we found that although total numbers of T cells are greatly reduced in *IL-7Rα*$^{-/-}$ mice, the percentage of Tregs relative to other T cell subsets was not affected (Fig. 5*A*) (8). Thus, IL7Rα signaling is not required for Treg development. However, it remains possible that IL-2Rβ and IL-7Rα can act redundantly to drive Treg development. To test this possibility, we compared Treg differentiation in *IL-7Rα*$^{-/-}$ vs *IL-2Rβ*$^{-/-}$ × *IL-7Rα*$^{-/-}$ mice. We found that *IL-2Rβ*$^{-/-}$ × *IL-7Rα*$^{-/-}$ mice showed a significant decrease in Treg numbers when compared with *IL-7Rα*$^{-/-}$ mice (p = 0.009, Student's t test) (Fig. 5, *A* and *B*). Importantly, the numbers of Tregs found in *IL-2Rβ*$^{-/-}$ × *IL-7Rα*$^{-/-}$ mice (thymus = 195 ± 75; spleen = 686 ± 136) were comparable to that which we observed in age-matched γc$^{-/-}$ mice (thymus = 80 ± 22; spleen = 1500 ± 651) in our previous studies (Fig. 5*B*) (8). These experiments demonstrate that IL-2Rβ and IL-7Rα-dependent cytokines are the only γc-dependent cytokines required for Treg development.

IL-7Rα and IL-15Rα are expressed at quite low levels on mature splenic Tregs. Thus, it is rather surprising that splenic Tregs are maintained in young *IL-2*$^{-/-}$ mice. To examine this further, we stained CD4$^+$Foxp3$^+$ Tregs from LMC and *IL-2*$^{-/-}$ mice for the expression of IL-7Rα and IL-15Rα. We found that CD4$^+$Foxp3$^+$

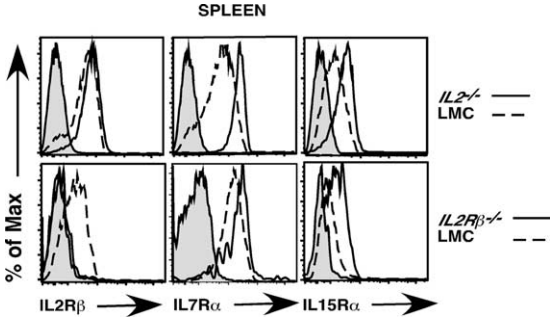

FIGURE 6. Expression of IL-2Rβ, IL-7Rα, and IL-15Rα on CD4$^+$Foxp3$^+$ Tregs from *IL-2*$^{-/-}$ and *IL-2Rβ*$^{-/-}$ mice compared with wild type LMC. Splenocytes were isolated from 4- to 5-wk-old LMC, *IL-2*$^{-/-}$ and *IL-2Rβ*$^{-/-}$ mice and stained with Abs for CD4, CD8, and Foxp3 to identify splenic Tregs. Shown are CD4$^+$Foxp3$^+$ gated cells stained for IL-2Rβ (*left panels*), IL-7Rα (*middle panels*), and IL-15Rα (*right panels*). Gray histograms represent staining of CD4$^+$Foxp3$^+$ cells with isotype control Ab. Solid lines represent histograms of CD4$^+$Foxp3$^+$ T cells from *IL-2*$^{-/-}$ (*top panel*) or *IL-2Rβ*$^{-/-}$ (*bottom panel*) mice; broken lines represent staining of CD4$^+$Foxp3$^+$ Tregs from wild-type LMC mice. A representative example of three independent experiments is depicted (n = 6 *IL-2*$^{-/-}$ and 3 *IL-2Rβ*$^{-/-}$ mice).

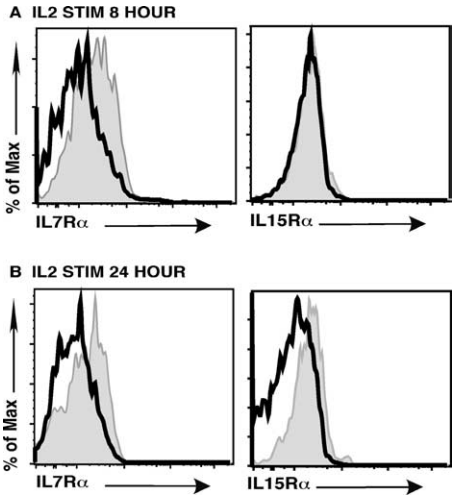

FIGURE 7. Expression of IL-7Rα and IL-15Rα on $CD4^+Foxp3^+$ Tregs in *IL-2*$^{-/-}$ mice after ex vivo IL2 stimulation. $CD4^+$ T cells were isolated as described in the *Materials and Methods* section and cells were stimulated with 100 U/ml IL-2 for 8 or 24 h. Cells were then stained for Foxp3, IL-7Rα, and IL-15Rα and analyzed by flow cytometry. Dark unshaded histogram represents IL-7Rα and IL-15Rα expression after IL-2 stimulation for the times indicated. Shaded histograms represent nonstimulated controls. Shown is a representative example of six independent experiments for IL7Rα expression and two independent experiments for IL15Rα expression.

Tregs in *IL-2*$^{-/-}$ mice expressed significantly higher levels of both the IL-7Rα and IL-15Rα chains (Fig. 6). We considered two explanations for these findings. First, it is possible that, in the absence of IL-2, any splenic Tregs that express higher levels of IL-7Rα or IL-15Rα have a competitive advantage and are selectively expanded. Alternatively, it is possible that IL-2/IL-2Rβ-dependent signals actively down-regulate IL-7Rα or IL-15Rα expression. To distinguish between these two possibilities, we stained the few $CD4^+Foxp3^+$ Tregs in *IL-2Rβ*$^{-/-}$ mice for IL-7Rα and IL-15Rα expression. Once again, we observed increased expression of both IL-7Rα and IL-15Rα on Tregs from *IL-2Rβ*$^{-/-}$ vs LMC mice (Fig. 6). In *IL-2Rβ*$^{-/-}$ mice, IL-15Rα expression provides no competitive advantage. Thus, this latter finding strongly suggests that IL-15Rα down-regulation, and likely IL-7Rα as well, is due to IL-2Rβ-dependent signals. To investigate this further, we took $CD4^+$ splenocytes from *IL-2*$^{-/-}$ mice and stimulated those cells with IL-2. We then examined expression of IL-7Rα and IL-15Rα chains on $CD4^+Foxp3^+$ cells (Fig. 7). We observed down-regulation of the IL-7Rα-chain as early as 8 h after IL-2 stimulation; both the IL-7Rα and IL-15Rα chains were clearly down regulated after 24 h of IL-2 stimulation. Taken together, these studies indicate that IL-2 dependent signals can negatively regulate IL-7Rα and IL-15Rα expression on $CD4^+Foxp3^+$ Tregs.

Discussion

These studies identify IL-2, IL-7, and IL-15 as the sole γc-dependent cytokines required for Treg development in the thymus. Four pieces of data support this conclusion. First, receptors for these three cytokines are expressed on developing Tregs in the thymus. Second, these cells can respond to IL-2 and IL-7 by inducing robust STAT5 activation. The only other γc-dependent cytokine receptors expressed on thymic Tregs are IL-4Rα and IL-21Rα. However, IL-4 and IL-21 did not induce STAT5 activation in $CD4^+Foxp3^+$ thymocytes and only induced minimal STAT5 activation in mature $CD4^+Foxp3^+$ splenocytes. The role of IL-15 is somewhat more complicated. We observed only weak STAT5 induction following ex vivo stimulation of thymic $CD4^+Foxp3^+$ Tregs with IL-15. This may reflect the absence of accessory cells in our ex vivo stimulation cultures that would allow for effective trans presentation of IL-15 to developing Tregs. Third, IL-2 and to a lesser degree IL-7 and IL-15 were capable of inducing the conversion of $CD4^+CD25^+Foxp3^-$ Treg progenitors into mature $Foxp3^+$ Tregs. In contrast, TSLP was completely ineffective in this assay. Finally, we found that *IL-2Rβ*$^{-/-}$ × *IL-7Rα*$^{-/-}$ mice recapitulated the phenotype reported in *γc*$^{-/-}$ mice which are essentially devoid of Tregs. In contrast, the reduction in thymic Tregs in *IL-2Rβ*$^{-/-}$ × *IL-4Rα*$^{-/-}$ mice was no more severe than that seen in *IL-2Rβ*$^{-/-}$ mice. Taken together with our previous observation that *IL-2*$^{-/-}$ × *IL-15*$^{-/-}$ mice have significantly fewer Tregs that *IL-2*$^{-/-}$ mice, our findings strongly support the conclusion that IL-2, IL-7, and IL-15, but not other γc-dependent cytokines, can contribute to Treg differentiation in the thymus.

We also examined the role of TSLP on Treg differentiation as previous studies have suggested that TSLP plays an important role in human and murine Treg development (17, 22). Our studies rule out a direct role for TSLP in murine Treg differentiation. First, consistent with our earlier observation, *IL-7Rα*$^{-/-}$ mice show no reduction in Tregs relative to non-Tregs. Second, we could not detect expression of TSLPRα on thymic Tregs nor induce STAT5 activation following stimulation of these cells with TSLP. Third TSLP was incapable of inducing the conversion of thymic Treg progenitors into $CD4^+Foxp3^+$ Tregs. These findings demonstrate that TSLP cannot play a direct role in Treg development. It remains possible that TSLP plays an indirect role by acting on other cell types that may be involved in promoting Treg differentiation. However, this function is either not unique to TSLP, or not critical, as Tregs clearly develop in mice lacking the IL-7Rα-chain, which is a critical component of the TSLPR.

Although our studies demonstrate that IL-2, IL-7, and IL-15 can redundantly contribute to Treg development and homeostasis, it seems likely that IL-2 is the relevant cytokine in wild-type mice. Specifically, we found that expression of IL-7Rα and IL-15Rα were significantly increased on Tregs in both *IL-2*$^{-/-}$ and *IL-2Rβ*$^{-/-}$ mice. Ex vivo stimulation of $CD4^+Foxp3^+$ Tregs from *IL-2*$^{-/-}$ mice with IL-2 led to rapid down-regulation of both of these receptor subunits. Thus, IL-2 plays an important role in rendering $CD4^+Foxp3^+$ Tregs uniquely responsive to IL-2-dependent signals in wild-type mice. This most likely serves to link Treg homeostasis directly to effector T cell activation and IL2 secretion. Effector T cell IL2 production appears to be critical for Tregs to expand in step with activated effector T cells and thereby mediate effective suppression. Supporting this conclusion, *IL-2*$^{-/-}$ mice, but not *IL-7*$^{-/-}$, or *IL-15*$^{-/-}$ mice, show signs of T cell activation and ultimately succumb to lethal multiorgan autoimmune disease. Thus, although IL-7 and IL-15 are capable of sustaining Treg populations in young mice, they are not effective at expanding these cells sufficiently during ongoing immune responses. Finally, these findings also have implications for the use of low-level IL-7Rα expression to identify Tregs (24, 25) as this receptor may be up-regulated under conditions of limited IL-2 availability.

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Disclosures

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